REMARKS

Telephone Interview

Applicants would like to express their appreciation to Examiner Chernyshev and Examiner Eyler for the courtesy extended to Applicants' agent, Angela Dallas Sebor, during the telephone interview on October 2, 2003. During the interview, the outstanding issues under 35 U.S.C. § 112, first paragraph were discussed. Examiner Eyler indicated that the Examiners would be withdrawing finality and reopening prosecution to reconsider issues under 35 U.S.C. § 112, first paragraph, on the basis of written description. During the interview, possible claim amendments were discussed that may place the claims in a condition for allowance or at least reduce issues upon the reopening of prosecution. In particular, the Examiner suggested that structural and more specific functional language be provided in the claims to meet the requirements for written description. Also, Applicants' agent indicated that a reference would be provided to demonstrate that one of skill in the art at the time of the invention would possess sufficient knowledge regarding the structure of FGF-2 to understand where modifications could be made to the protein without destroying functional activity of the protein. The Examiners indicated that any amendments presented in response to the final Office Action would be entered and considered, given the proposal to reopen prosecution.

Claim Amendments:

The claims have been amended in a manner discussed by the Examiner. Specifically, the independent Claim 1 has been amended to recite specific structural and functional limitations for the FGF-2 protein portion of the chimera, as well as for the penetratin peptide portion of the chimera. All of the amendments are supported in the original specification and claims as filed. The limitations of Claims 4 and 9 and from the specification on page 17, lines 11-16 have been imported into Claim 1 and Claims 4 and 9 have been cancelled, without prejudice to or disclaimer of the subject matter therein. Support for the more specific functional language regarding FGF-2 is found in the specification on page 13, lines 11-27.

The structure-function relationship of FGF-2 proteins as known in the art and described in the specification is discussed in detail below under the response to the rejection under 35 U.S.C. § 112, first paragraph. With regard to the penetratin peptide portion of the chimeric protein, Applicants submit that the specification and the art at the time of the invention provide a detailed description of the structure and function of the peptide, such that one of skill in the art would be able to recognize that the inventors were in possession of the invention as claimed at the time of filing.

Specifically, Applicants refer to the specification at page 10, lines 3-14; page 20, line 9 to page 23, line 14, where penetratins are discussed both structurally and functionally in detail, including *multiple* examples of penetratin sequences useful in the invention from a variety of sources, and including reference to several publications which further describe such sequences. The motif described on page 21 and recited in the claims describes for the skilled practitioner what structural features are required of a penetratin peptide and the specification further teaches the function of the peptide and how to ascertain whether a peptide has such function (e.g., see Example 3).

Objection to the Specification and Rejection of Claim 4 Under 35 U.S.C. § 112, First Paragraph:

The Examiner has objected to the specification and rejected Claim 4 under 35 U.S.C. § 112, first paragraph, on the basis of written description. Specifically, the Examiner contends that the specification fails to describe the entire genus of proteins encompassed by the claim, which recites proteins that are at least 70% identical to the reference FGF-2 sequence. The Examiner also asserts that the claims recites a chimeric FGF protein that is 70% identical to an FGF-2 sequence, but fails to indicate other relevant identifying characteristics, such as physical and/or chemical and/or functional characteristics coupled with a known or disclosed correlation between structure and function.

Applicants traverse the rejection of Claim 4 under 35 U.S.C. § 112, first paragraph. Initially, it is noted that Claim 4 has been cancelled and therefore, the rejection of Claim 4 is moot. However, the limitations of Claim 4 have been added to Claim 1 and so the rejection will be addressed with regard to Claim 1 and as discussed in the October 2 telephone interview.

First, Applicants wish to clarify that, contrary to how the Examiner describes Claim 4, the claim does not reference the entire FGF chimeric protein with regard to the percent identity. Rather, the claim recites the *portion* of the chimeric protein that has FGF-2 activity with regard to the percent identity.

Second, it is submitted that the specification, combined with the knowledge in the art at the time of the invention, provides sufficient guidance regarding the structure and function of FGF-2 proteins that one of skill in the art would readily be able to identify and produce homologues of FGF-2 proteins that have the recited identity to the reference sequence and retain FGF-2 biological activity, such that it is clear that the inventors were in possession of the claimed invention at the time of filing. First, with regard to the function of FGF proteins, the specification provides a detailed discussion of the biological activities of FGF proteins, including references to a variety of specific

art-recognized assays for measuring such activity (e.g., see page 2, line 3 to page 3, line 20; and page 13). In addition, the specification describes two specific FGF-2 sequences (SEQ ID NO:5 (bovine) and SEQ ID NO:6 (human), teaches that numerous FGF proteins are known in the art and share similar requirements for FGF receptor binding and heparan sulfate dependence, for example, and teaches methods for modifying FGF-2 proteins and for determining percent identity (e.g., page 15, lines 14-27; page 17, line 17 through page 20, line 8).

Moreover, as discussed in the October 2 interview, the structure of FGF-2 proteins from many animal species was well known in the art at the time of the invention and significant knowledge was available regarding which amino acid residues are conserved among species and which amino acids contribute to the biological activities of FGF-2 (i.e., a structure-to-function relationship), such that one of skill in the art would readily be able to produce a modified FGF-2 protein that retains biological activity.

In support of Applicants' position, enclosed herewith are two publications which describe many details of the structure of FGF-2 which are correlated with the protein function and which were known in the art at the time of the invention. First, referring to the enclosed review article by Ornitz and Itoh (*Genome Biology* 2(3):3005.1-3005.12, 2001), this review shows that FGF proteins "have been intensely studied for nearly 30 years" (page 6, col. 2). The review makes reference to studies on the characteristic structure correlated with function of FGF proteins, including specifically FGF-2 (page 2-3; and Fig. 3), and it is noted that much of this information, including the three dimensional structure of FGF-2 was known prior to the present invention. The Ornitz reference cites Coulier et al., which is the second enclosed reference.

More specifically, Coulier et al. (*J. Mol. Evol.* 44:43-56, 1997) illustrates in great detail the conserved structure of FGF proteins, and specifically indicates what amino acids in the structure are correlated with function. For example, Coulier et al. provide an amino acid alignment of sequences from 40 different FGF species, including eight FGF-2 species (see Fig. 1), and describe/illustrate which amino acids are absolutely conserved among all FGF proteins, which amino acids are absolutely conserved among all the FGF-2 proteins, which amino acids are similar between all FGF or FGF-2 proteins, and finally, provide a discussion of which regions of the protein correlate to specific FGF functions (see page 45 through page 47). This reference shows that 10% of residues (12 residues of the 120 amino acid core sequence) are conserved among all FGF proteins, with highly conserved positions (e.g., positions 79-89) and regions with limited similarity (e.g., positions 97-114 or 138-146). Coulier et al. mention important amino acids that have been correlated with

function, including two cysteine residues, the receptor binding site, and the heparan sulfate binding site, as well as important residues for saccharide binding. It is further noted that among FGF-2 species, Table 1 shows that the eight amino acid sequences for FGF-2 have differences that produce identities across species between 84% and 98%, and it is submitted that one of skill in the art would readily be able to make additional modifications to the sequences given the clear structure-to-function correlation established by just this reference and predictably produce a functional synthetic variant of the human or bovine FGF-2 sequences, which are the recited sequences. Therefore, it is submitted that Applicants are entitled to the breadth of claims as recited, since the specification, in combination with the knowledge in the art, provide significant detail regarding the structure-to-function relationship for FGF-2.

In view of the foregoing remarks, Applicants respectfully request that the Examiner withdraw the rejection of Claim 4 under 35 U.S.C. § 112, first paragraph.

Applicants have attempted to address all of the issues as set forth in the July 10 Office Action and as discussed in the October 2 telephone interview. In the event that the Examiner has any additional questions or concerns regarding the present claims, she is encouraged to contact the below-named agent at (303) 863-9700.

Respectfully submitted,

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Date: October 9, 2003

J Mol Evol (1997) 44:43–56

JOURNAL OF MOLECULAR EVOLUTION

Of Worms and Men: An Evolutionary Perspective on the Fibroblast Growth Factor (FGF) and FGF Receptor Families

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Received: 6 April 1996 / Accepted: 5 July 1996

Abstract. FGFs (fibroblast growth factors) play major roles in a number of developmental processes. Recent studies of several human disorders, and concurrent analysis of gene knock-out and properties of the corresponding recombinant proteins have shown that FGFs and their receptors are prominently involved in the development of the skeletal system in mammals. We have compared the sequences of the nine known mammalian FGFs, FGFs from other vertebrates, and three additional sequences that we extracted from existing databases: two human FGF sequences that we tentatively designated FGF10 and FGF11, and an FGF sequence from *Cænorhabditis elegans*. Similarly, we have compared the sequences of the four FGF receptor paralogs found in chordates with four non-chordate FGF receptors, including one recently identified in *C. elegans*. The comparison of FGF and FGF receptor sequences in vertebrates and nonvertebrates shows that the FGF and FGF receptor families have evolved through phases of gene duplications, one of which may have coincided with the emergence of vertebrates, in relation with their new system of body scaffold.

Key words: FGF — FGF receptor — Phylogeny — Vertebrate — Invertebrate — Gene duplication

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Introduction

A man may fish with the worm that hath eat of a king, and eat of the fish that hath fed of that worm.
Shakespeare, *Hamlet*

Comparative genomic and developmental analyses may provide clues for understanding the origin of genes as well as help in linking macro-evolutionary morphological transformations to modifications in embryonic patterns of expression of specialized genes (Sordino et al. 1995; Coates 1995). We compiled available data on the FGF (historically, fibroblast growth factors) and FGF receptor (FGFR) gene families in various organisms, and we review these data in an evolutionary context. In mammals, the FGF family currently comprises 11 members which interact with membrane-associated tyrosine kinase receptors (FGFR1 to FGFR4), and with heparan-sulfate proteoglycans. FGFs/FGFRs interactions play major roles in various developmental processes involving formation of mesoderm during gastrulation, integration of growth, budding and patterning during early postimplantation, and development of various tissues such as ear, limb, hair, and the skeletal system (Johnson and Williams 1993; Mason 1994; Wilkie et al. 1995; Yamaguchi and Rossant 1995). These roles have been established through study of patterns of expression, gene invalidations, physiological experiments using application of beads soaked with recombinant proteins, and analysis of human hereditary skeletal disorders (Johnson et al. 1994; Niswander et al. 1994; Muenke and Schell 1995; Tanaka

and Gann 1995; Tickle 1995; Yamaguchi and Rossant 1995). Two new human *FGF* genes, and for the first time an invertebrate (*C. elegans*) sequence that has the potential to code for an FGF-related molecule, have been identified in databases (Wilson et al. 1994; Hodgkin et al. 1995), confirmed by more extensive DNA sequencing, and added to the existing list of FGF members. Recently, a *C. elegans* sequence encoding an FGF receptor was reported (DeVore et al. 1995). The discovery of FGF and FGF receptor–encoding genes in worms allows for speculation both on the role of such factor-receptor interactions in nonvertebrates and on the evolution of the families.

In line with the proposed function of FGF/FGFR interactions in the development of the skeletal system, we suggest that an important increase in the number of *FGF* genes might be associated with the period of macroevolutionary change that coincided with the origin of vertebrates and might have thus provided information in the making of the skeletal system.

Methods

Sequences. Protein sequences were obtained directly from EMBL, NCBI (Genbank and dbEST), or Swissprot databases. When needed, nucleotide sequences (also obtained from databases) were translated using the PCGene (Intelligenetics) package. Additional sequencing was done by automated methods using Applied Biosystem 373A instrument.

Species abbreviations are as follows: bt: *Bos taurus* (bovine); cc: *Coturnix coturnix* (quail); ce: *Caenorhabditis elegans* (nematode); dm: *Drosophila melanogaster* (fruit fly); dr: *Danio rerio* (zebrafish); gd: *Gallus domesticus* (chicken); hs: *Homo sapiens* (human); ma: *Mesocricetus auratus* (golden hamster); md: *monodelphis domestica* (short-tailed opossum); mm: *Mus musculus* (mouse); nv: *Notophtalmus viridescens* (newt); oa: *Ovis aries* (sheep); ol: *Oryzias latipes* (medaka fish); pw: *Pleurodeles waltl* (Iberian ribbed newt); rn: *Rattus norvegicus* (rat); sp: *Stroxyglocentrotus purpuratus* (purple urchin); ss: *Sus scrofa* (pig); xl: *Xenopus laevis* (African clawed frog).

Accession numbers for FGF and FGFR sequences are as follows: bt-FGF1: P03968; bt-FGF2: P03969; bt-FGF4: P48803; cc-FGFR4: X76885; ce-FGF: U00048.; ce-FGFR: U39761; dm-FGFRa: Q07407; dm-FGFRb: Q09147; dr-FGF3: P48802; dr-FGFR4: U23839; gd-FGF1: A60130; gd-FGF2: P48800; gd-FGF3: P48801; gd-FGF4: P48804; gd-FGFR1: M24637; gd-FGFR2: M35196; gd-FGFR3: M35195; hs-FGF1: P05230; hs-FGF2: P09038; hs-FGF3: P11487; hs-FGF4: P08620; hs-FGF5: P12034; hs-FGF6: P10767; hs-FGF7: P21781; hs-FGF8: g999172; hs-FGF9: P31371; hs-FGF10: Z70275, T27215, H15590; hs-FGF11: Z70276, H19128, H62672, R58169, H28811; hs-FGFR1: P11362; hs-FGFR2: P21802; hs-FGFR3: P22607; hs-FGFR4: P22455; hs-IL1b: P01584; hs-IL1R: P14778; hs-SRC: P12931; ma-FGF1: P34004; md-FGF2: P48798; mm-FGF2: P15655; mm-FGF4: P11403; mm-FGF5: P15656; mm-FGF6: P21658; mm-FGF7: P36363; mm-FGF8: P37237; nv-FGFR2: L19870; oa-FGF2: P20003; oa-FGF7:P48808; ol-FGFR1: D13550; ol-FGFR2: D13551; ol-FGFR3: D13552; ol-FGFR4: D13553; pw-FGFR1: X59380; pw-FGFR2:X74332; pw-FGFR3: X75603; pw-FGFR4: X65059; rn-FGF1: P10935; rn-FGF2: P13109; rn-FGF5: P48807; rn-FGF7: Q02195; rn-FGF9: P36364; sp-FGFR: U17164; ss-FGF1: JH0476; xl-FGF2: P12226; xl-FGF3: P36386; xl-FGF4-I: P48805; xl-FGF4-II: P48806; xl-FGFR1: M61687; xl-FGFR2: X65943; xl-FGFR4: D31761.

The hs-FGF10 sequence was obtained by assembling EMBL database sequences H15590 and T27215, along with extensions and corrections from additional sequencing of the corresponding clones, ym27b06 and MT0120, respectively.

The hs-FGF11 sequence was obtained by assembling EMBL database sequences H19128, H62672, R58169, and H28811, along with extensions and corrections from additional sequencing of clone ym44e12 corresponding to H19128.

The hs-FGF10 and hs-FGF11 core sequences have been submitted to the EMBL database and assigned accession numbers Z70275 and Z70276, respectively.

Sequence Alignment. All protein sequences were aligned using the Clustal W program (Thompson et al. 1994).

FGF "core" sequences corresponding to hs-FGF1 amino acids 28–151, after removing internal insertions in FGF1 (corresponding to amino acids 120 and 121 of hs-FGF1), FGF3 (corresponding to amino acids 137–152 of hs-FGF3), FGF5 (corresponding to amino acids 181–186 of hs-FGF5), FGF7 (corresponding to amino acids 159–162 of hs-FGF7), FGF9 (corresponding to amino acids 156–161 of hs-FGF9), hs-FGF10 (amino acids 92–97 of the putative partial sequence), hs-FGF11 (amino acids 92–97 of the putative partial sequence), and ce-FGF (amino acids 92–95 of the putative sequence), were used for the alignment.

FGFR extracellular domain sequences (acidic box, Ig loops II and III) corresponding to amino acids 119–359 of hs-FGFR1 were aligned after removing internal insertions in ce-FGFR (amino acids 459–465) and sp-FGFR (amino acids 441–444 and 453–481).

FGFR intracellular domain sequences (kinase subdomains II–VII; Hanks et al. 1988 corresponding to amino acids 492–663 of hs-FGFR1) were aligned after removing internal insertions in ce-FGFR (amino acids 747–750 and762–773), dm-FGFRa (amino acids 519–522), and dm-FGFRb (amino acids 823–829).

Phylogeny Inference. Phylogenetic trees were constructed using the distance matrix (Dayhoff PAM matrix) and neighbor-joining algorithms of the Phylogeny Inference Package of J. Felsenstein (Felsenstein 1989). Human Interleukin 1β (hs-IL1b), human interleukin 1 β-receptor (hs-IL1R), and human c-SRC (hs-SRC) sequences were used as outgroups in the constructions of trees for the FGFs and the FGFRs ecto- and kinase domains, respectively.

Bootstrapping is a resampling technique that allows one to calculate confidence limits on trees (Felsenstein 1985). The bootstrap value (in percentages) indicates the number of times a given branching occurs among the bootstrapped samples and is a measure of the significance of a grouping with respect to the particular data set and to the method used for drawing the tree (Higgins et al. 1991). To test the validity (robustness) of branching, a total of 2,000 bootstrapped data sets were subjected to analysis, and a consensus tree was obtained using the Consense program (Felsenstein 1989).

Results

Identification of New FGF Genes FGF10, FGF11, and ce-FGF

Database searches with known FGF sequences allowed us to identify FGF-related human cDNA clones (ym27b06, yr45d03, ym44e12 (Hillier et al. 1996), and MTO120 (Brody et al. 1995)), isolated as Expressed Sequence Tag (EST), and a cosmid clone (CO5D11) (Wilson et al. 1994), isolated from a *C. elegans* genomic library. Based on nucleic acid and amino acid alignments of the partial sequences, we were able to identify the

Table 1. Amino acid identity score between FGF sequences

	ce-FGF	hs-FGF11	hs-FGF10	m-FGF9	hs-FGF9	mm-FGF8	hs-FGF8	m-FGF7	oa-FGF7	mm-FGF7	hs-FGF7	mm-FGF6	hs-FGF6	m-FGF5	mm-FGF5	hs-FGF5	xl-FGF4-II	xl-FGF4-I	mmFGF4	gd-FGF4	bt-FGF4	hs-FGF4	xl-FGF3	gd-FGF3	br-FGF3	hs-FGF3	xl-FGF2	m-FGF2	oa-FGF2	mm-FGF2	md-FGF2	gd-FGF2	bt-FGF2	hs-FGF2	ss-FGF1	m-FGF1	ma-FGF1	gd-FGF1	bt-FGF1
hs-FGF1	31	35	35	42	42	24	24	38	40	39	40	32	34	40	40	40	37	39	36	36	38	39	40	40	43	38	55	57	57	57	56	57	57	57	98	97	98	91	93
bt-FGF1	32	36	34	40	40	24	24	36	37	37	37	32	34	40	40	40	37	39	36	37	38	39	40	40	42	38	53	56	57	56	55	57	57	56	90	90	91	88	
gd-FGF1	30	34	35	42	42	23	23	38	39	39	39	32	34	40	40	40	37	39	37	36	40	40	40	41	44	38	54	57	57	57	56	57	57	57	90	89	89		
ma-FGF1	32	36	34	42	42	25	25	38	40	39	40	33	35	41	41	40	37	39	36	37	39	40	40	40	43	38	56	57	58	57	57	57	58	57	97	99			
m-FGF1	32	36	35	42	42	25	25	38	40	40	40	33	35	41	41	40	37	39	36	37	39	40	40	40	43	38	55	57	57	57	56	57	57	57	97				
ss-FGF1	33	35	35	42	42	24	24	37	39	38	39	32	34	40	40	40	36	38	36	36	38	39	40	40	42	39	54	56	57	56	55	56	57	56					
hs-FGF2	30	34	33	41	41	30	30	39	40	40	40	45	45	45	45	44	40	41	42	45	44	45	43	44	45	44	86	98	98	98	94	92	98						
bt-FGF2	31	34	33	41	41	30	30	38	39	39	39	44	44	45	45	44	40	41	42	45	44	45	43	44	45	44	86	98	100	98	94	93							
gd-FGF2	32	31	32	40	40	28	28	38	39	39	39	44	45	44	44	43	40	42	42	43	43	44	40	41	43	42	85	93	93	93	92								
md-FGF2	29	33	32	40	40	30	30	39	40	40	40	44	45	43	43	42	40	42	40	44	41	43	42	43	45	43	84	95	94	95									
mm-FGF2	30	34	33	41	41	30	30	39	40	40	40	44	45	44	44	43	40	42	41	44	43	44	43	44	45	44	85	100	98										
oa-FGF2	31	34	33	41	41	30	30	38	39	39	39	44	44	45	45	44	40	41	42	45	44	45	43	44	45	44	86	98											
m-FGF2	30	34	33	41	41	30	30	39	40	40	40	44	45	44	44	43	40	42	41	44	43	44	43	44	45	44	85												
xl-FGF2	28	33	34	39	39	26	26	36	36	36	36	41	40	42	42	41	40	40	40	41	42	43	40	40	41	41													
hs-FGF3	36	36	37	47	47	35	35	45	47	46	47	42	42	51	51	50	40	40	34	42	36	36	87	89	78														
br-FGF3	34	36	35	46	46	33	33	45	46	45	46	43	43	50	50	50	42	42	34	43	37	37	83	83															
gd-FGF3	38	37	37	50	50	35	35	47	49	48	49	42	42	51	51	50	40	40	34	41	35	35	95																
xl-FGF3	36	37	36	50	50	35	35	46	48	47	48	41	41	49	49	48	39	39	33	40	34	34																	
hs-FGF4	31	43	44	44	44	32	32	34	35	36	36	68	69	51	51	51	75	76	91	80	90																		
bt-FGF4	28	40	43	42	42	30	30	32	32	33	32	69	70	51	51	51	69	70	89	75																			
gd-FGF4	31	43	39	43	43	36	36	35	36	37	36	76	78	49	49	50	84	85	77																				
mm-FGF4	27	41	41	41	41	32	32	32	32	33	33	66	67	48	48	48	71	73																					
xl-FGF4-I	30	44	42	43	43	36	36	35	36	37	36	72	74	48	48	49	97																						
xl-FGF4-II	29	43	40	42	42	35	35	35	36	37	36	73	75	48	48	49																							
hs-FGF5	35	37	38	47	47	31	31	42	44	43	44	52	51	97	97																								
mm-FGF5	36	36	38	48	48	31	31	43	45	44	45	51	50	100																									
m-FGF5	36	36	38	48	48	31	31	43	45	44	45	51	50																										
hs-FGF6	31	40	38	43	43	36	36	36	38	37	38	94																											
mm-FGF6	31	41	39	43	43	35	35	34	36	35	36																												
hs-FGF7	36	34	35	43	43	31	31	93	98	98																													
mm-FGF7	35	35	36	43	43	31	31	95	97																														
oa-FGF7	36	34	35	43	43	31	31	93																															
m-FGF7	34	33	35	43	43	31	31																																
hs-FGF8	28	22	27	36	36	100																																	
mm-FGF8	28	22	27	36	36																																		
hs-FGF9	38	39	40	100																																			
m-FGF9	38	39	40																																				
hs-FGF10	32	66																																					
hs-FGF11	30																																						

human clones as two new *FGF* sequences, tentatively called *hs-FGF10* (ym27b06 and MTO120) and *hs-FGF11* (yr45d03 and ym44e12). Further sequencing of these clones allowed us to derive the peptide sequences for the complete core region (Coulier et al. 1991, 1994). *hs-FGF10*- and *hs-FGF11*-related genomic sequences appear to exist in the mouse (data not shown).

Z. Du and R. Waterston (unpublished) predicted the existence of a gene encoding a heparin-binding growth-factor-related peptide within cosmid CO5D11 derived from chromosome III of *C. elegans*. Alignment of this peptide sequence, tentatively called ce-FGF, with that of known FGFs, revealed 27–37% of amino acids identity with the other members of the family. We have determined that mRNA corresponding to this gene can be found in larval stages of *C. elegans* (data not shown).

FGF Sequence Comparisons Show Conserved and Variable Stretches of Amino Acids

The core amino acid sequences of 39 identified vertebrate FGFs (corresponding to 11 mammalian, four avian, one fish, and three amphibian FGF paralogs) and the *C. elegans* FGF (ce-FGF) were aligned for comparison (Table 1; Fig. 1). The core sequence was obtained by deletion of N- and C-terminal extensions as well as specific internal sequences. It represents about 120 amino acid residues in length. Twelve positions (10%) were found conserved in all sequences. The conservation among the other residues varies along the core sequence, with scattered clusters of highly conserved positions (for example, 79–89) and regions with limited similarity (for example, 97–114 or 138–146).

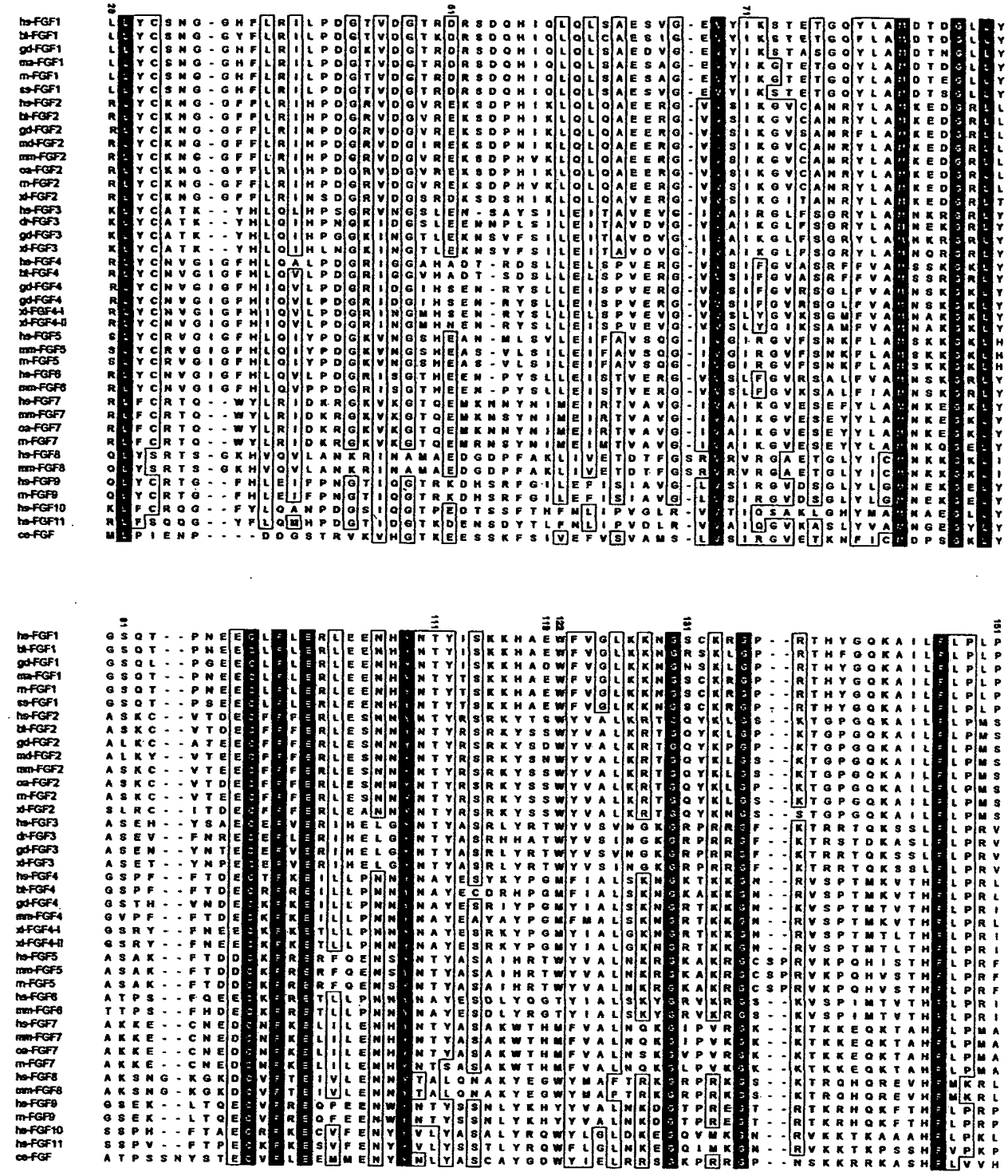


Fig. 1. Alignment of FGF sequences. Sequences derived from 40 known FGF proteins were aligned, allowing for gaps (–) in order to optimize the alignment. Positions of perfect identity are indicated (*black boxes*), while *open boxes* contain amino acids that are identical or similar in at least eight orthologous sequences. Amino acid numbering is according to human FGF1 sequence.

Earlier studies on FGFs have tried to define structure–function relationships using mutants or protein fragments (Jaye et al. 1986; Presta et al. 1992). The determination of the three-dimensional structure of FGF1 and FGF2 has brought clues about the importance of some amino acid residues (Eriksson et al. 1991; Zhang et al. 1991; Zhu et al. 1991; Faham et al. 1996). These include the role of the cysteine residues and the position of the heparan-sulfate– and receptor-binding sites. The two cysteines consistently present in FGF sequences do not seem

Table 2. Amino acid identity score between FGFR sequences

	Kinase domain																									
	sp-FGFR	dm-FGFRb	dm-FGFRa	ce-FGFR	xl-FGFR4	pw-FGFR4	ol-FGFR4	hs-FGFR4	dr-FGFR4	cc-FGFR4	pw-FGFR3	ol-FGFR3	hs-FGFR3	gd-FGFR3	xl-FGFR2	pw-FGFR2	ol-FGFR2	nv-FGFR2	hs-FGFR2	gd-FGFR2	xl-FGFR1	pw-FGFR1	ol-FGFR1	hs-FGFR1	gd-FGFR1
gd-FGFR1	66	58	60	54	76	74	81	73	79	76	85	84	82	87	78	84	85	85	86	85	92	93	90	97		
hs-FGFR1	66	58	60	54	75	73	79	72	77	75	84	83	82	85	76	82	84	83	84	83	90	92	90		96	
ol-FGFR1	65	56	60	54	73	69	76	70	76	72	80	80	77	81	77	80	83	80	83	81	88	92				
pw-FGFR1	67	58	59	54	74	70	77	72	77	73	82	83	79	84	77	80	84	81	83	82	92			86	87	
xl-FGFR1	67	57	58	54	76	72	77	72	76	72	81	83	78	83	77	79	83	80	82	81		78		79	81	
gd-FGFR2	65	58	61	51	77	74	81	74	79	78	88	86	87	90	90	97	91	97	99		71	74		74	75	
hs-FGFR2	65	58	61	51	76	74	80	73	78	77	88	86	85	90	89	95	92	96		93	70	75		73	74	
nv-FGFR2	65	58	61	51	75	73	80	73	78	77	87	86	85	89	87	98	90		86	87	69	71		72	73	
ol-FGFR2	65	57	59	52	77	76	83	74	81	78	87	87	84	89	86	89										
pw-FGFR2	64	58	61	51	75	73	79	73	77	77	87	85	85	88	87			94	86	85	69	72		73	73	
xl-FGFR2	62	58	60	52	76	71	77	73	77	77	81	83	83	83		83		83	82	81	66	68		68	69	
gd-FGFR3	66	58	62	55	78	78	86	77	84	80	97	95	94		70	74		75	79	79	70	76		74	75	
hs-FGFR3	66	59	63	54	79	76	82	77	81	78	91	91		88	68	71		71	74	74	67	69		70	71	
ol-FGFR3	66	58	61	54	77	76	83	74	83	78	94															
pw-FGFR3	65	58	62	55	77	77	85	74	83	78			84	87	66	70		70	73	72	66	69		69	70	
cc-FGFR4	63	57	57	50	80	82	83	78	82		73		76	76	65	69		67	69	70	62	64		65	64	
dr-FGFR4	62	55	60	50	80	81	95	74		75	69		72	73	66	69		68	68	69	64	62		63	64	
hs-FGFR4	62	56	60	52	76	74	75		71	80	70		70	72	64	66		65	66	69	63	63		64	64	
ol-FGFR4	63	55	60	50	82	83																				
pw-FGFR4	59	53	55	49	83			78	76	84	72		77	75	64	68		67	68	69	63	64		65	65	
xl-FGFR4	64	56	58	52		79		77	75	83	73		76	76	66	69		67	70	70	64	64		66	67	
ce-FGFR	51	48	50		30	31		32	31	29	32		31	34	32	34		34	34	34	34	32		33	32	
dm-FGFRa	56	77		30	35	34		34	33	36	35		36	38	33	33		33	34	35	34	33		33	33	
dm-FGFRb	54		33	31	29	29		30	29	30	33		31	33	31	30		30	33	31	30	31		29	29	
sp-FGFR		28	28	27	29	28		31	31	29	35		33	35	33	32		34	33	34	32	34		34	34	
	Extracellular domain																									

to form intramolecular bonds. Indeed, one of the cysteines (position 98 in Fig. 1) is conserved in all FGFs, but the other (position 31) is present in most FGFs but not in FGF8, FGF11, and ce-FGF. The presumed receptor-binding site of FGF2 has been located in the region delimited by residues 106–121. The differences in amino acid sequences within this stretch (which includes insertions of up to 16 residues in FGF3) could account for the discrimination between different receptors. Basic residues K32, K127, R128, K133, K137, and K143 (as numbered in Fig. 1), are thought to constitute a potential binding site for the sulfate group of heparin and other sulfated substrates (Eriksson et al. 1991; Zhang et al. 1991; Zhu et al. 1991; Faham et al. 1996). Other residues possibly involved in saccharide binding include N33, N107, and Q142 (Faham et al. 1996). They are relatively well conserved in all FGF sequences except for K32, N33, K127, and K143.

FGFR Sequence Comparisons

The sequences of 25 FGF receptors identified in various species, including recently published FGFR sequence from *C. elegans* (DeVore et al. 1995), were aligned for comparison (Table 2 and Fig. 2). Comparisons were done separately to the extracellular and kinase domains but led for similar conclusions. In vertebrates, FGFR1, FGFR2, and FGFR3 orthologous sequences have more than 80% identity. FGFR4 sequences are slightly more different. The four identified invertebrate sequences, including the two *Drosophila* FGFR, share an average of 30% identity in the extracellular domain, and from 49 to 67% in the kinase domain, with FGFR1 to FGFR4 vertebrate sequences, and thus could not be identified as orthologs of any of them.

The extracellular regions of the FGF receptors contain 22 amino acid residues conserved throughout all sequences (Fig. 2A). This includes cysteine residues involved in the tertiary structure of immunoglobulin-like domains. Some of these residues, such as C278, Y340, C342, and S347 of FGFR2, are targets of mutations found in human inherited skeletal disorders leading to receptor dysfunction (Muenke and Schell 1995; Mulvihill 1995; Wilkie et al. 1995; Yamaguchi and Rossant 1995). However, several mutations occur in residues which are not conserved in invertebrates. One residue that is not conserved in invertebrate FGFRs is a proline

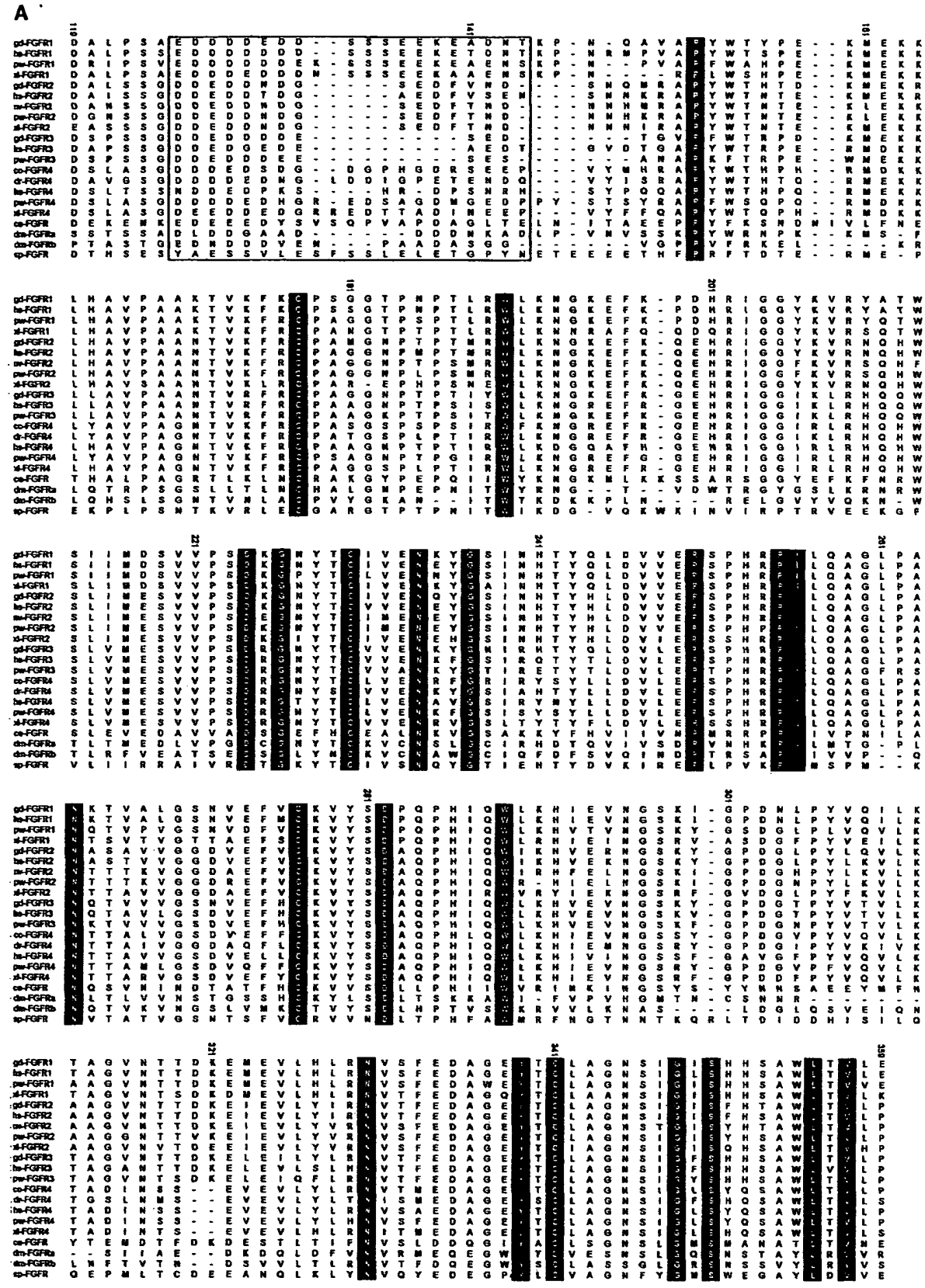


Fig. 2. Alignment of FGFR sequences. A Alignment of extracellular domains. Sequences derived from the extracellular domain of 21 known FGF-Receptor proteins were aligned, allowing for gaps (–) in order to optimize the alignment. Portions of sequences used for the alignment include the acidic box and the two C-terminal Ig loops. Positions of perfect identity are indicated (*black boxes*). *Open box* indicates the position of the acidic domain. Amino acid numbering is according to human FGFR1 sequence.B Alignment of kinase domains. Sequences derived from the kinase domain of 25 known FGF-Receptor proteins were aligned, allowing for gaps (–) in order to optimize the alignment. Portions of sequences used for the alignment include kinase subdomains II–VII (Hanks et al. 1988). Positions of perfect identity are indicated (*black boxes*). Amino acid numbering is according to human FGFR1 sequence.

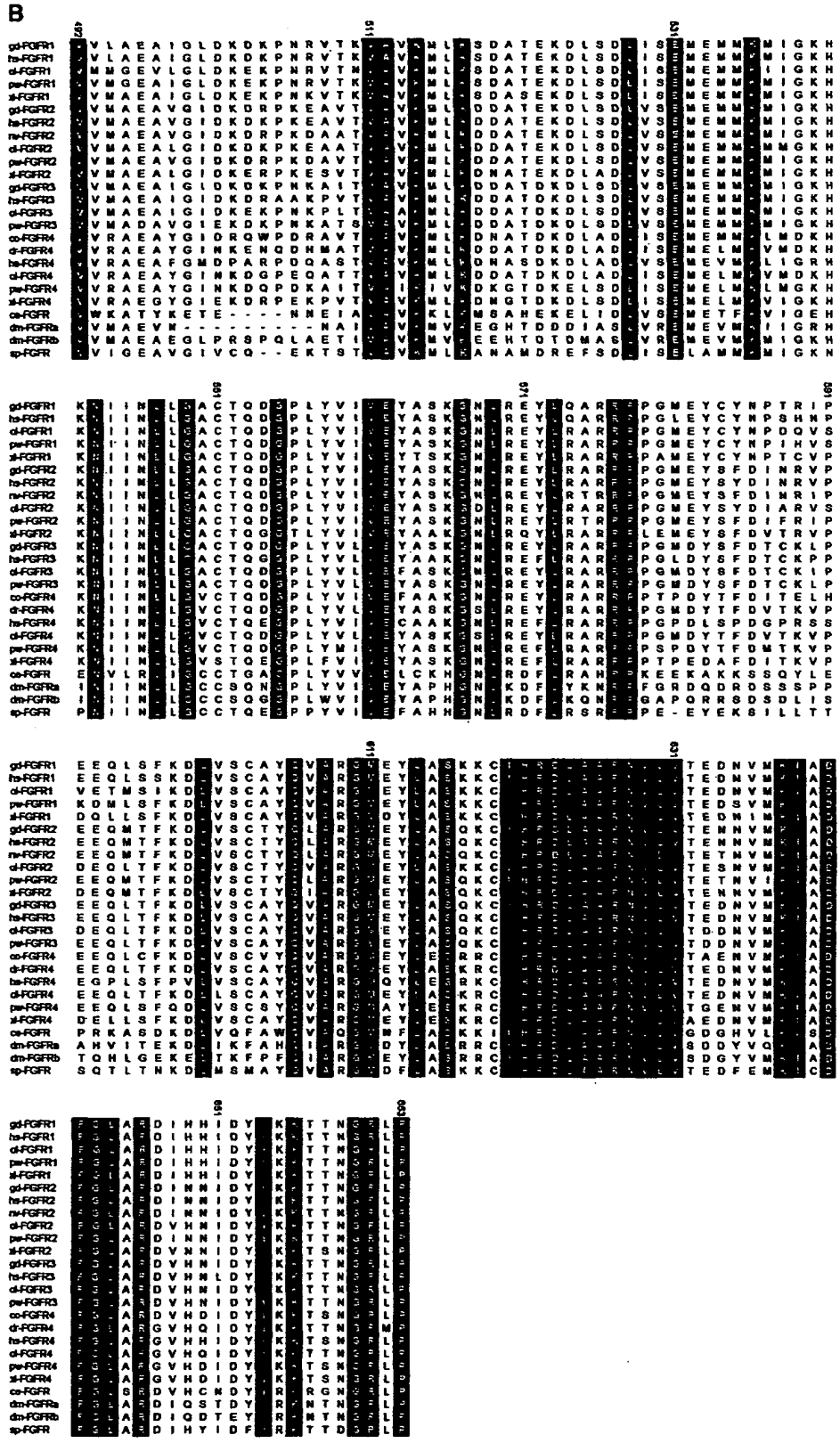
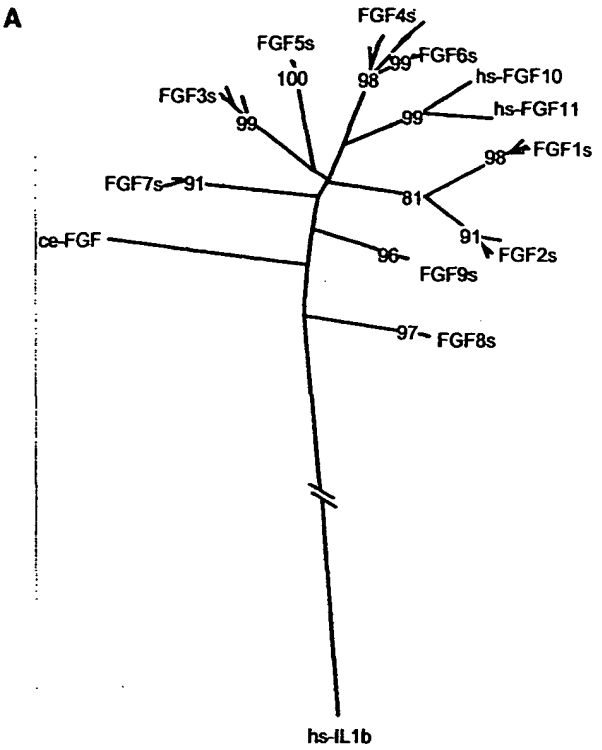
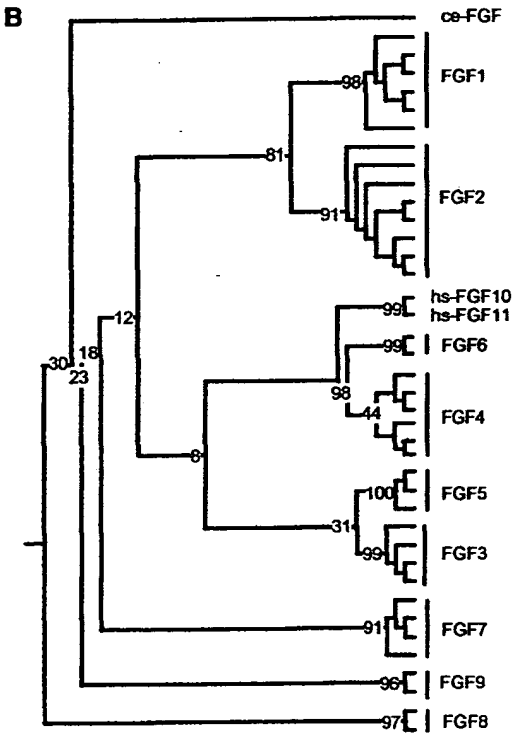


Fig. 2. Continued.

(position 252 in FGFR1 and 253 in FGFR2) (Fig. 2). Substitution of this proline by arginine in FGFR1 and FGFR2 is found in two craniosynostosis syndromes, the Pfeiffer and Apert syndromes, respectively. Interestingly, an arginine residue is found at the corresponding position in the *C. elegans* FGFR sequence. The point mutations found in human disorders are dominant and are presumed to activate constitutively the receptor. It





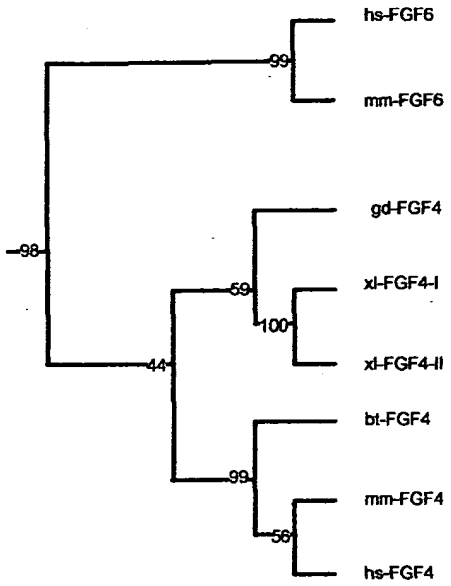


Fig. 3. Phylogenetic analysis of the FGF family. The 40 sequences aligned in Fig. 1, including mammalian, avian, batracian, and invertebrate FGF sequences, and human interleukin 1-β as an outgroup (*hs-IL 1b*), were used to infer a phylogenetic tree. A Phylogenetic tree where branch lengths are grossly proportional to calculated genetic distances, except for hs-IL 1b. Only the bootstrap values higher than 75% are indicated. B Phenogram representation of the inferred phylogenetic tree, and close-up view of the FGF4/FGF6 groups. Branch lengths are arbitrary. Bootstrap values are indicated for all the nodes that group together FGF paralogs.

would be interesting to test whether the counter mutation in *C. elegans* (i.e., arginine to proline) leads to a change in ce-FGFR activity.

Evolution of the FGF Family

The 40 sequences aligned in Fig. 1 were used to infer a phylogenetic tree (Fig. 3) by using the neighbor-joining algorithm (Felsenstein 1989). The sequence of human interleukin-1β, a peptide regulatory factor distantly related to FGFs (Gimenez-Gallego et al. 1985; Eriksson et al. 1991; Zhang et al. 1991; Zhu et al. 1991), was used as an outgroup. Use of the parsimony algorithm led to a similar topology.

All paralogs were grouped together; bootstrap values exceeded 90%.

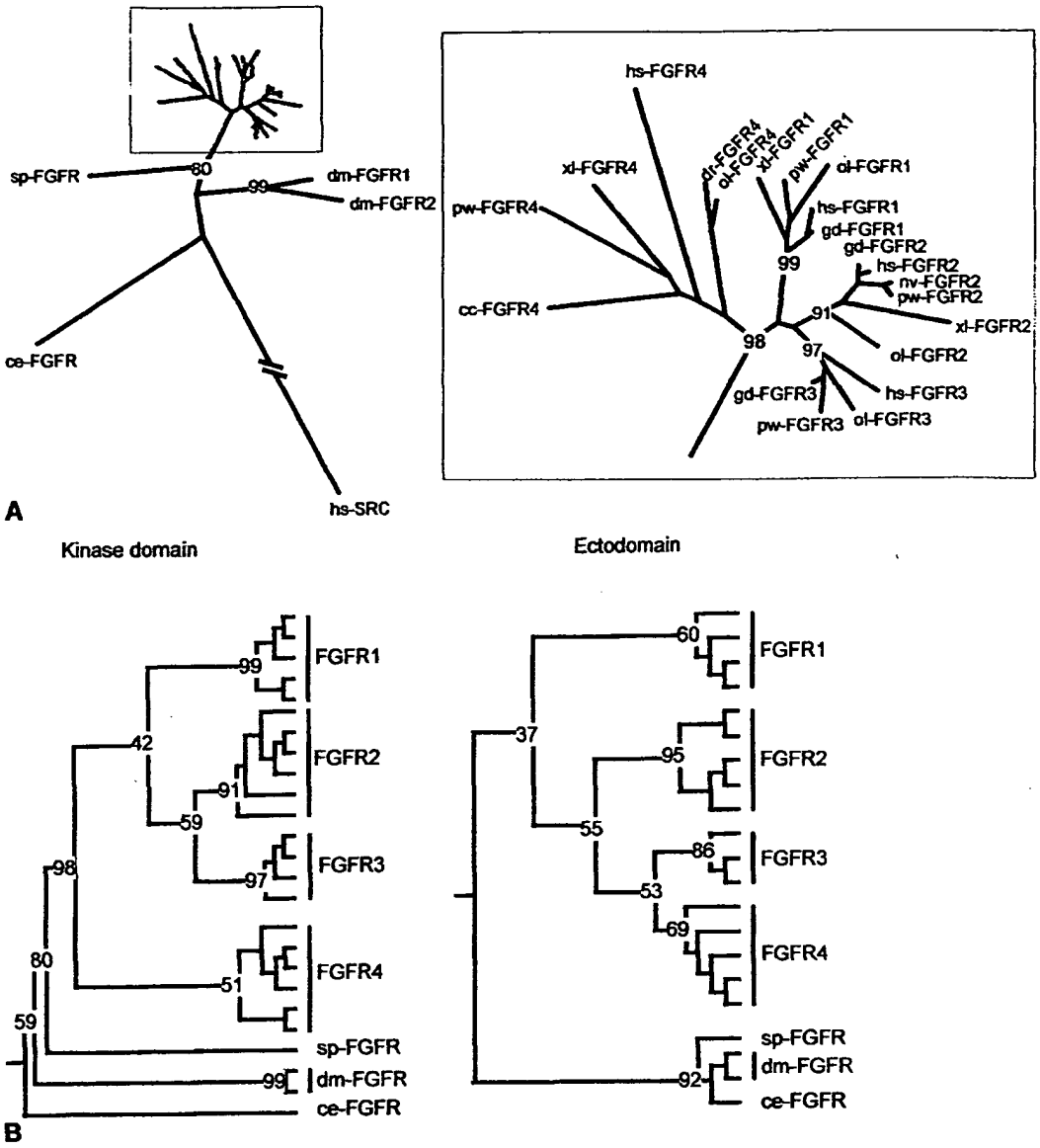


Fig. 4. Phylogenetic analysis of the FGFR family. The 25 kinase domains sequences aligned in Fig. 2B, including mammalian, avian, amphibian, fish, and invertebrate FGFR sequences, and human c-SRC as an outgroup (*hs-SRC*), were used to infer a phylogenetic tree. A Phylogenetic tree where branch lengths are proportional to calculated-genetic distances, except for hs-SRC.A closer view of the vertebrate FGFR subtree is shown on the right. Only the bootstrap values higher than 75% are indicated. B Phenogram representation of inferred phylogenetic tree using either FGFR kinase or ectodomains. Branch lengths are arbitrary. Bootstrap values are indicated for all the nodes that group together FGFR paralogs.

Studying the branching between paralogs showed that the only branchings with high confidence intervals were between FGF1 and FGF2 (bootstrap value of 81%, see Materials and Methods section), FGF4 and FGF6 (98%), and FGF10 and FGF11 (99%). None of the other branchings between paralogs could exceed a bootstrap value of 31%. FGF1/FGF2, FGF4/FGF6, and FGF10/FGF11 are pairs of FGFs that show the highest similarity score (Table 1), suggesting the corresponding pairs of genes duplicated more recently as compared to the other *FGF* genes.

Evolution of the FGF Receptor Family

A similar analysis was conducted with FGF receptor sequences (Fig. 4). Separate analyses of the extracellular region and intracellular kinase domain were performed using both neighbor-joining and parsimony algorithms, and yielded similar topologies (Fig. 4B). Comparison with the corresponding sequence alignments (Table 2) showed that changes in the kinase domain have occurred at a much slower rate.

Invertebrate FGFRs from three species appeared in separate branches which were all distinct from the vertebrate sequences (Fig. 4A). FGFR paralogs from vertebrate species were grouped together with high bootstrap values. The two FGFRs from *D. melanogaster* were distinct from vertebrate FGFRs and branched together with a very high value of bootstrap (99%), suggestive of a very recent duplication. This topology is in support of the

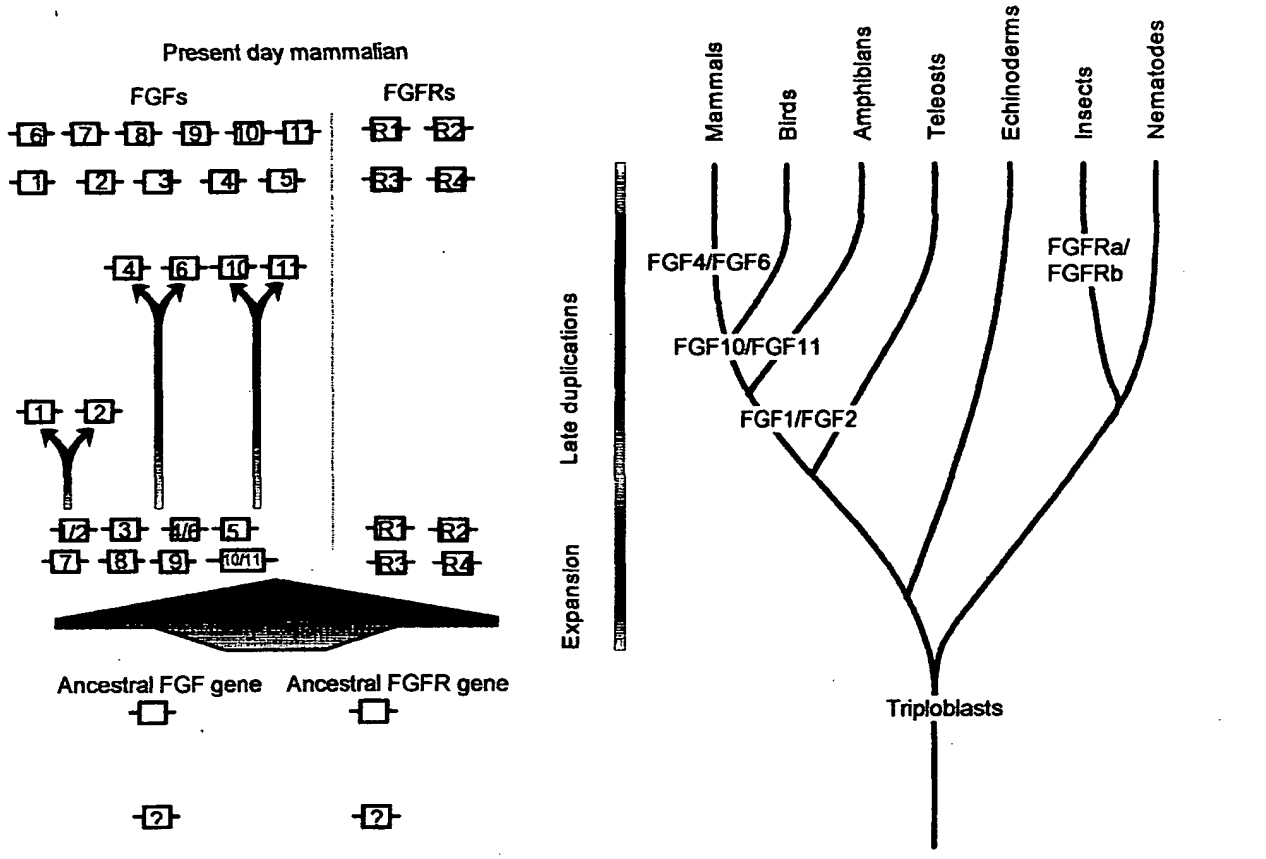


Fig. 5. Representation of a hypothetical FGF and FGFR evolution scheme. This evolutionary scheme is based on the available information on FGF and FGFR sequences in various species. Putative phases of gene duplications (shown on the left) are individualized, and are tentatively related toa phylogenetic tree of Metazoa (shown on the right). FGF and FGFR expansion is shown to occur after the origin of nematodes and echinoderms, but the timing of this expansion will only be precisely fixed upon determination of the number of FGF and FGFR sequences in these species.

existence of one FGFR gene (or a recently duplicated one like in *D. melanogaster*) in invertebrates and of an expansion to four members in vertebrates.

Discussion

This paper reports the presence of an FGF gene in an invertebrate species, the identification of two new human FGF, and the comparison of their deduced amino acid sequences with the known members of the FGF family. A similar analysis was done with FGFR sequences from invertebrate and vertebrate species. Phylogenetic trees inferred from the calculated genetic distances allow for hypotheses concerning the timing and functional significance of *FGF* and *FGFR* gene-family expansion and divergence.

Hypotheses for the Origin of the Topologies

Several hypotheses may explain the observed topologies. It is possible to individualize a few steps in a series of duplications from a common ancestor at the origin of the present-day members of the *FGF* family. The most recent steps gave rise to *FGF4* and *FGF6* and to *FGF10* and *FGF11*. *FGF1* and *FGF2* may have been created at a similar period, or slightly before *FGF4/FGF6* and *FGF10/FGF11* divergences. We call this step the phase of late duplications (Fig. 5). A more ancient series of duplications, occurring over a limited time period, led to the emergence of eight members—namely, *FGF3, FGF5, FGF7, FGF8, FGF9,* and the *FGF1/FGF2, FGF4/FGF6,* and *FGF10/FGF11* putative ancestors. The topology of the tree suggests that these eight members could have derived from a single gene; the emergence of several FGFs would have occurred during a phase of major genome expansion. The topology of the FGFR tree suggests the existence of an ancestral FGFR gene and only one phase of expansion, leading to four FGFR members as identified in vertebrates.

When did the FGF expansion occur with respect to the origin of the branch leading to nematodes?

The expansion could have occurred before the separation between protostomia and deuterostomia, and up to eight FGFs may be represented in nematodes. If this is the case, it could mean that other putative *ce-FGF* genes may exist and be related to a particular mammalian FGF. Interestingly, a number of genes from chromosome 17 in humans and chromosome III in *C. elegans* (Ruddle et al. 1994) are presumed to be homologs, suggesting that *ce-FGF* and *hs-FGF10* might be paralogs.

FGF/FGFR Expansion May Be Associated with the Origin of Vertebrates

Alternatively, the FGF expansion could have occurred after the protostomia/deuterostomia separation (Fig. 5). One can assume, from the fraction of sequenced genomic DNA and cDNAs from *C. elegans,* that about 30% of the total number of its genes are currently known (Wilson et al. 1994; Berks and The C. elegans Genome Mapping and Sequencing Consortium 1995; Hodgkin et al. 1995). Assuming both a random distribution of FGF-encoding genes throughout the *C. elegans* genome and good efficiency in the search strategies used here, this restricts the number of potential *FGF* genes in this species. It is thus unlikely that eight genes, orthologous to each *FGF* and to the *FGF1/FGF2, FGF4/FGF6,* and *FGF10/FGF11* ancestors, are present in *C. elegans.* A likely hypothesis, sufficient to explain the evolution of the FGF family, is that FGF expansion occurred after the separation of deuterostomia and protostomia and was contemporaneous with a phase of global gene duplications that took place during the period leading to vertebrate emergence (Holland et al. 1994). Confirmation of this hypothesis will await identification of more FGF sequences in the invertebrates.

The topology of the FGFR tree is strongly in support of this hypothesis if one assumes the two families have coevolved. The full complexity of the FGF receptor system already exists in amphibia (Thisse et al. 1995) and bony fish (Emori et al. 1992) as a probable coevolution with its ligand family, but not in insects or echinoderms. Current failure to identify any FGF in *Drosophila* could be due to technical reasons, or, alternatively, be due to an evolutionary process which has resulted in the loss or absence of this type of gene in insects. Insects are known to have evolved as a separate branch of the metazoan tree distant from chordates (Fig. 5). The fact that FGF receptors were found in *Drosophila* (Klämbt et al. 1992; Shishido et al. 1993) would appear to argue against the latter hypothesis but does not constitute definitive proof. In a similar manner, tyrosine kinase neurotrophin receptors have been identified in the fly, but their activation occurs by way of homophylic interaction and is independent of ligands (Pulido et al. 1992). In any case, the FGFRs characterized in *Drosophila* are different from the vertebrate FGFRs (Shishido et al. 1993). It is probable that the full complexity of the family, as it exists in mammals, is not developed in this species. Thus the low number or even complete absence of FGFs in insects is consistent with our hypothesis of FGF expansion associated with the origin of vertebrates.

It is interesting to note that FGFs play important roles in the development of the skeletal system, as shown by the characterization of mutations in their receptors in inherited human diseases. Mutations of FGFR1, FGFR2, and FGFR3 lead to disorders of the long bones and of the flat bones of the skull associated with achondroplasia and craniosynostosis, respectively (Muenke and Schell 1995; Wilkie et al. 1995). (It is interesting to note that FGFR4 behaves slightly differently from the other three receptors in several ways, including its topology in the tree and its noninvolvement in human inherited diseases.) Moreover, studies of limb bud growth and sclerotome formation have demonstrated the important role of FGFs in this process (Tickle 1995; Tanaka and Gann 1995; Grass et al. 1996). It is therefore tempting to speculate that expansion of the FGF/FGFR families is associated with the emergence of the vertebrate systems of motricity.

Possible Role for the Late Duplication Events

When did the phase of late duplications take place? Identification of FGF4 and FGF6 or of their ancestor in fishes, amphibia, and birds could set the time for this step, providing they represent all phyla, without loss through extinction.

The crucial role of FGF4 in limb bud development could suggest that the *FGF4–FGF6* duplication event originated as a consequence of the generation of the morphological novelty that is the tetrapod limb during the fin-to-limb transition (Nelson and Tabin 1995), and that it took place after the origin of the fish lineage. This hypothesis suggests that *FGF4* and *FGF6* orthologs may actually exist in amphibia, but this is not firmly established. The identification of four *FGF* genes in frog and bird and the analysis of the phylogenetic tree suggest that *FGF* genes orthologous to the mammalian *FGFs* exist in these species. Are there amphibian or avian genes orthologous to each mammalian gene or to only eight of them? In other words, did the last step of duplication, which created *FGF1* and *FGF2, FGF4* and *FGF6,* and *FGF10* and *FGF11,* occur before or after the origin of amphibia and birds? Four *FGF* genes have been isolated in frog (Isaacs et al. 1992). Two of these sequences, designated *xl-FGF4-I* and *xl-FGF4-II* and corresponding to pseudo-alleles, are highly related to both *FGF4* and *FGF6* (75–76% and 74–75% amino acid identity with hs-FGF4 and hs-FGF6, respectively). A growth factor, related to both FGF4 and FGF6, has been characterized in chicken (Niswander et al. 1994). Like the above-mentioned *Xenopus FGF, gd-FGF4* seems only slightly more related to *FGF4* than to *FGF6* (Table 1—80% and 78% identity with hs-FGF4 and hs-FGF6, respectively). Thus *FGF4* and *FGF6* orthologs may not exist in birds. It is thus possible to speculate that the FGF4/FGF6 duplication may have occurred after the separation of the bird/reptile branch. While the inferred tree groups together mammal and nonmammal FGF4 (Fig. 3A,B), it should be noted that this grouping has a very low bootstrap value (44%) and may not be significant. Interestingly, FGF4 function is essential for early postimplantation events in the mouse (Feldman et al. 1995), a role not relevant to birds or amphibia.

Table 3. Chromosomal localization of genes of the *FGF* family in humans and the mouse

Gene	Chromosomal localization in: Humans	Mouse	References
FGF1	5q31–33	18	(Jaye *et al.* 1986; Cox *et al.* 1991)
FGF2	4q26–27	3 A2–B	(Lafage-Pochitaloff *et al.* 1990; Mattéi *et al.* 1992; Cox *et al.* 1991)
FGF3	11q13	7 F	(Casey *et al.* 1986; Peters *et al.* 1984)
FGF4	11q13	7 F	(Adélaïde *et al.* 1988; Peters *et al.* 1989)
FGF5	4q21	5 E1–F	(Nguyen *et al.* 1988; Mattéi *et al.* 1992)
FGF6	12p13	6 F3–G1	(Marics *et al.* 1989; deLapeyrière *et al.* 1990)
FGF7	15	2 F–G	(Kelley *et al.* 1992; Mattéi *et al.* 1995a)
FGF8	10q25–q26	19 C3–D	(White et al. 1995; Mattéi *et al.* 1995a)
FGF9	13q12	–	(Mattéi *et al.* 1995b)
FGF10	–	–	
FGF11	–	–	

The presence of chick *FGF1* and *FGF2* genes indicates that the duplication of *FGF1/FGF2* occurred before the origin of the bird/reptile lineage. *xl-FGF2* sequence is more related to hs-FGF2 (86%) than to hs-FGF1 (55%), and the presence of FGF1 protein has been reported in *X. laevis* (Shiurba et al. 1991). The *FGF1/FGF2* duplication may therefore have occurred even before the origin of amphibia. The late duplication phase could thus be subdivided into two events (Fig. 5), the *FGF1/FGF2* duplication having occurred before the origin of amphibia and the *FGF4/FGF6* duplication as late as after the separation between birds and mammals.

As only the human *FGF10* and *FGF11* genes have been identified, there are no data upon which to speculate as to the timing of the *FGF10/FGF11* duplication. The failure to have detected either of these closely related *FGFs* prior to the search of sequence databases may be more than coincidental and reflect somewhat different functions of these two new *FGFs*.

In humans and in the mouse, two species in which chromosomal localization of the *FGF* genes has been determined, *FGFs* are located on different chromosomes. As an exception, *FGF3* and *FGF4* are tandemly linked on chromosomal band 11q13 in humans and on chromosome 7 in the mouse (Table 3). It could be noted also that *FGF4* and *FGF6* on chromosomes 11 and 12 on the one hand, and *FGF1* and *FGF2* on chromosomes 4 and 5 on the other hand, are, respectively, on pairs of chromosomes that appear to contain paralogous genes and are thought to derive from each other (Lundin 1993). The identification of remnants of genome evolution may be explained by late events of duplication. These events could be related to the *FGF* late duplication phase.

An Integrated View of FGF/FGFR Function and Evolution

The presence of an *FGF* gene in *C. elegans* allows for the hypothesis of a scheme of evolution before and after the protostomia/deuterostomia separation.

The biological role of an FGF protein in nematodes is open to investigation. It could be involved in mesoderm induction and mesoderm development or positioning. Studies of mutant *FGFR* gene in *C. elegans* suggest *FGF/FGFR* could be associated with development or positioning of the muscle system (DeVore et al. 1995). It is tempting to speculate that *FGFs* are contemporary of triploblast phyla and may not exist in diploblast species (Fig. 5).

Among various possible roles during embryogenesis, recent studies have shown that FGFs are involved in migration and patterning during the formation of the skeletal system of mammals. Failure to infer a robust phylogenetic tree from available data suggests the *FGF* genes have evolved considerably since their separation from a common ancestor and may be explained by a series of duplications occurring early in evolution. This expansion in the number of *FGF* genes may have been an important determinant of skeletal system formation in vertebrates. A second and late phase of *FGF* duplications may be related to the establishment of improved signaling networks—also involving *BMP, WNT, Hedgehog,* and *HOX* family members—responsible for the fin-to-limb transition or to the split between ray- and lobe-finned bony fish. This scheme is reminiscent of the presumed evolution of the homeobox-containing *HOX* genes, during which the amplification of a single cluster was coincidental with the transition from invertebrate chordates to vertebrates and was followed by the acquisition of extra *HOX* genes in relation to the appearance of vertebrate head and limb. The *FGF* and *HOX* gene families could have coevolved, leading ultimately to a higher complexity. More generally, the expansion of families of genes is presumed to have coincided with metazoan radiation. Further identification of *FGF* and *FGFR* genes in other organisms and comparison of their protein sequences are necessary to help confirm or refute the above hypothesis.

Note Added in Proof

While this manuscript was in press, Itoh and co-workers reported the isolation of a new FGF gene in rat, which

they called *Fgf10* [Yamasaki et al., *J. Biol Chem* (1966) 271:15918–15921]. The two new human FGF genes described in our paper should therefore be designated *FGF11* and *FGF12*. FGF homologous factor (FHF)-1 to -4 genes have also been reported recently [Smallwood et al., *Proc Natl Acad Sci USA* (1996) **93**:9850–9857]. *FHF-3* and *FHF-1* correspond to *FGF11* and *FGF12* described here. We propose *FHF-2* and *FHF-4* be designated *FGF13* and *FGF14*, in agreement with the recommendation of the Nomenclature Committee [Baird et al., *Ann NY Acad Sci* (1991) **638**:xiii–xxvi].

Acknowledgments. This work was supported by INSERM and by grants from the Fédération Nationale des Centres de Lutte Contre le Cancer, the Association pour la Recherche sur le Cancer, and the Ligue Nationale Contre le Cancer. We are grateful to the following scientists for their help and advice: M. Arpagaus, D. Baty, F. Birg, O. deLapeyrière, P. Dessen, P. Golstein, J. Izard, B. Jordan, M.-G. Mattei, C. Mawas, M.-J. Pébusque, S. Pizette, J.M. Plaza, D. Thierry-Mieg, A.-S. Verdier, and to the G.I.S. Infobiogen for computer support.

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Protein family review

Fibroblast growth factors

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Published: 9 March 2001

Genome Biology 2001, 2(3):reviews3005.1–3005.12

The electronic version of this article is the complete one and can be found online at http://genomebiology.com/2001/2/3/reviews/3005

Summary

Fibroblast growth factors (FGFs) make up a large family of polypeptide growth factors that are found in organisms ranging from nematodes to humans. In vertebrates, the 22 members of the FGF family range in molecular mass from 17 to 34 kDa and share 13-71% amino acid identity. Between vertebrate species, FGFs are highly conserved in both gene structure and amino-acid sequence. FGFs have a high affinity for heparan sulfate proteoglycans and require heparan sulfate to activate one of four cell-surface FGF receptors. During embryonic development, FGFs have diverse roles in regulating cell proliferation, migration and differentiation. In the adult organism, FGFs are homeostatic factors and function in tissue repair and response to injury. When inappropriately expressed, some FGFs can contribute to the pathogenesis of cancer. A subset of the FGF family, expressed in adult tissue, is important for neuronal signal transduction in the central and peripheral nervous systems.

Gene organization and evolutionary history

Gene organization

The prototypical *Fgf* genes contain three coding exons (Figure 1), with exon 1 containing the initiation methionine, but several *Fgf* genes (for example, *Fgf2* and *Fgf3*) have additional 5' transcribed sequence that initiates from upstream CUG codons [1,2]. The size of the coding portion of *Fgf* genes ranges from under 5 kb (in *Fgf3* and *Fgf4*) to over 100 kb (in *Fgf12*). In several *Fgf* subfamilies, exon 1 is subdivided into between two and four alternatively spliced subexons (denoted 1A-1D in the case of *Fgf8*). In these *Fgf* genes, a single initiation codon (ATG) in exon 1A is used. This gene organization is conserved in humans, mouse and zebrafish, but its functional consequences are poorly understood. Other subfamilies of *Fgfs* (such as *Fgf11-14*) have alternative amino termini, which result from the use of alternative 5' exons. It is not known whether a common 5' untranslated exon splices to these exons or whether alternative promoter and regulatory sequences are used.

Most *Fgf* genes are found scattered throughout the genome. In human, 22 *FGF* genes have been identified and the chromosomal locations of all except *FGF16* are known (Table 1) [3-7]. Several human *FGF* genes are clustered within the genome. *FGF3*, *FGF4* and *FGF19* are located on chromosome 11q13 and are separated by only 40 and 10 kb, respectively; *FGF6* and *FGF23* are located within 55 kb on chromosome 12p13; and *FGF17* and *FGF20* map to chromosome 8p21-p22. These gene locations indicate that the *FGF* gene family was generated both by gene and chromosomal duplication and translocation during evolution. Interestingly, a transcriptionally active portion of human *FGF7*, located on chromosome 15q13-q22, has been amplified to about 16 copies, which are dispersed throughout the human genome [8].

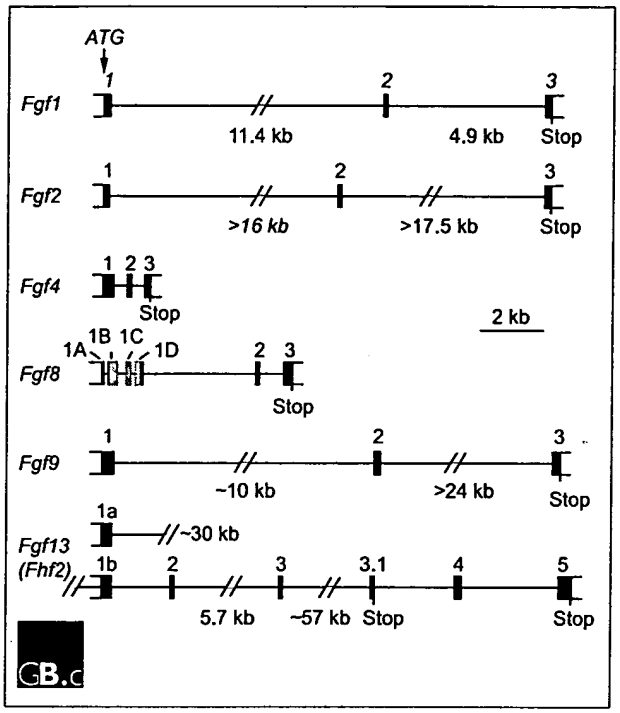

Figure 1
Gene structure of selected members of the *Fgf* family. Only the portion of each gene containing coding exons is shown. Constitutively expressed exons are in black; alternatively spliced exons are in gray. *Fgfs 1, 2, 4* and *9* contain the prototypic three-exon organization. For *Fgf1*, 5' untranslated exons are not shown; inclusion of these exons extends the gene by approximately 69 kb [78]. *Fgf8* is an example of a gene with 5' alternative splicing, and *Fgf13* demonstrates alternatively used 5' exons separated by over 30 kb. References: *Fgf1* [78]; *Fgf2* [79]; *Fgf4* [80]; *Fgf8* [52]; *Fgf9* [81]; *Fgf13* [76].

In the mouse, there are at least 22 *Fgf* genes [3,9], and the locations of 16 have been identified (Table 1). Many of the mouse *Fgf* genes are scattered throughout the genome, but as in the human, *Fgf3*, *Fgf4* and *Fgf19* are closely linked (within 80 kb on chromosome 7F) and *Fgf6* and *Fgf23* are closely linked on chromosome 6F3-G1.

Evolutionary history

Fgfs have been identified in both invertebrates and vertebrates [3]. Interestingly, an *Fgf*-like gene is also encoded in the nuclear polyhedrosis virus genome [10]. *Fgf*-like sequences have not been found in unicellular organisms such as *Escherichia coli* and *Saccharomyces cerevisiae*. Although the *Drosophila* and *Caenorhabditis elegans* genomes have been sequenced, only one *Fgf* gene (*branchless*) has been identified in *Drosophila* [11] and two (*egl-17* and *let-756*) have been identified in *C. elegans* [12,13], in contrast to the large number of *Fgf* genes identified in vertebrates. The evolutionary relationship between invertebrate and vertebrate *Fgfs* is shown in Figure 2a.

The *Fgf* gene expansion has been hypothesized to be coincident with a phase of global gene duplications that took place during the period leading to the emergence of vertebrates [14]. Across species, most orthologous FGF proteins are highly conserved and share greater than 90% amino-acid sequence identity (except human FGF15 and mouse Fgf19; see below). To date, four *Fgfs* (*Fgf3, 8, 17* and *18*) have been identified in zebrafish, seven (*Fgf3, Fgf(i), Fgf(ii), Fgf8, 9* and *20*) in *Xenopus* (*Fgf(i) and Fgf(ii)* are most closely related to *Fgf4* and *Fgf6* [15]) and seven (*Fgf2, 4, 8, 12, 14, 18* and *19*) in chicken [3].

The apparent evolutionary relationships of the 22 known human FGFs are shown in Figure 2b. Vertebrate FGFs can be classified into several subgroups or subfamilies. Members of a subgroup of FGFs share increased sequence similarity and biochemical and developmental properties. For example, members of the FGF8 subfamily (FGF8, FGF17, and FGF18) have 70-80% amino acid sequence identity, similar receptor-binding properties and some overlapping sites of expression (for example, the midbrain-hindbrain junction) [16,17]. Members of FGF subgroups are not closely linked in the genome, however, indicating that the subfamilies were generated by gene-translocation or by genome-duplication events, not by local duplication events.

Human *FGF15* and mouse *Fgf19* have not been identified. Human *FGF19* is evolutionarily most closely related to mouse *Fgf15* (51% amino acid identity; Figure 2b) [18] and both the human *FGF19* and mouse *Fgf15* genes are closely linked to the human and mouse *Fgf3* and *Fgf4* genes on orthologous regions of human chromosome 11q13 and mouse chromosome 7F (N.I., unpublished observations). These findings indicate that human *FGF19* may be the human ortholog of mouse *Fgf15*. Because all other *Fgf* orthologs share greater than 90% amino acid identity, it remains possible that the true orthologs of these genes have not been identified, have been lost or have diverged during vertebrate evolution.

Characteristic structural features

FGFs range in molecular weight from 17 to 34 kDa in vertebrates, whereas the *Drosophila* FGF is 84 kDa. Most FGFs share an internal core region of similarity, with 28 highly conserved and six identical amino-acid residues [19]. Ten of these highly conserved residues interact with the FGF receptor (FGFR) [20]. Structural studies on FGF1 and FGF2 identify 12 antiparallel β strands in the conserved core region of the protein (Figure 3) [21,22]. FGF1 and FGF2 have a β trefoil structure that contains four-stranded β sheets arranged in a triangular array (Figure 3b; reviewed in [23]). Two β strands (strands β10 and β11) contain several basic amino-acid residues that form the primary heparin-binding site on FGF2. Regions thought to be involved in receptor binding are distinct from regions that bind heparin (Figure 3) [21-24].

Table 1

Chromosomal localizations of FGFs in human and mouse

Human		Mouse		References	Accession numbers	
Gene	Location	Gene	Location		Human	Mouse
FGF1	5q31	*Fgf1*	18	[82,83]	X65778, E03692, E04557	U67610, M30641
FGF2	4q26-27	*Fgf2*	3A2-B	[84,85]	E05628, M27968	M30644, AF065903, AF065904, AF065905
FGF3	11q13	*Fgf3*	7F	[86-88]	X14445	Y00848
FGF4	11q13.3	*Fgf4*	7F	[87,89]	E03343	M30642
FGF5	4q21	*Fgf5*	5E1-F	[85,90]	M37825	M30643
FGF6	12p13	*Fgf6*	6F3-G1	[91,92]	X63454	M92416
FGF7	15q15-21.1	*Fgf7*	2F-G	[93,94]	M60828	Z22703
FGF8	10q24	*Fgf8*	19C3-D	[54,95]	U36223, U56978	Z48746
FGF9	13q11-q12	*Fgf9*	14D	[81,96,97]	D14838	U33535, D38258
FGF10	5p12-p13	*Fgf10*	13A3-A4	[98,99]	AB002097	D89080
FGF11 (FHF3)	17p13.1	*Fgf11*	-	[100]	U66199	U66203
FGF12 (FHF1)	3q28	*Fgf12*	16B1-B3	[31,100-102]	U66197	U66201
FGF13 (FHF2)	Xq26	*Fgf13*	X	[31,76,103]	U66198	U66202, AF020737
FGF14 (FHF4)	13q34	*Fgf14*	14	[31]	U66200	U66204
-		*Fgf15**	7F	(N.I., unpublished observations)		AF007268
FGF16	-	*Fgf16*	-		AB009391	AB049219
FGF17	8p21	*Fgf17*	14	[104]	AB009249	AB009250
FGF18	5q34	*Fgf18*	-	[105]	AB007422, AF075292	AB004639, AF075291
*FGF19**	11q13.1	-		[106]	AB018122, AF110400	
FGF20	8p21.3-p22	*Fgf20*	-	[27,107]	AB030648, AB044277	AB049218
FGF21	19q13.1-qter	*Fgf21*	-	[108]	AB021975	AB025718
FGF22	19p13.3	*Fgf22*	-	[109]	AB021925	AB036765
FGF23	12p13.3	*Fgf23*	6F3-G1	[7,75] (N.I., unpublished)	AB037973, AF263537	AB037889, AF263536

*Human *FGF19* and mouse *Fgf15* may be orthologous genes.

Localization and function

Localization

Subcellular localization and secretion

Most FGFs (FGFs 3-8, 10, 15, 17-19, and 21-23) have amino-terminal signal peptides and are readily secreted from cells. FGFs 9, 16 and 20 lack an obvious amino-terminal signal peptide but are nevertheless secreted [25-27]. FGF1 and FGF2 also lack signal sequences, but, unlike FGF9, are not secreted; they can, however, be found on the cell surface and within the extracellular matrix. FGF1 and FGF2 may be released from damaged cells or could be released by an exocytotic mechanism that is independent of the endoplasmic-reticulum-Golgi pathway [28]. FGF9 has been shown to contain a non-cleaved amino-terminal hydrophobic sequence that is required for secretion [29,30]. A third subset of FGFs (FGF11-14) lack signal sequences and are thought to remain intracellular [31-34]. It is not known whether these FGFs interact with known FGFRs or function in a receptor-independent manner within the cell. FGF2 and FGF3 have high-molecular-weight forms that arise from initiation from upstream CUG codons [2,14,35]. The additional amino-terminal sequence in these proteins contains nuclear-localization signals, and the proteins can be found in the nucleus; the biological function of nuclear-localized FGF is unclear.

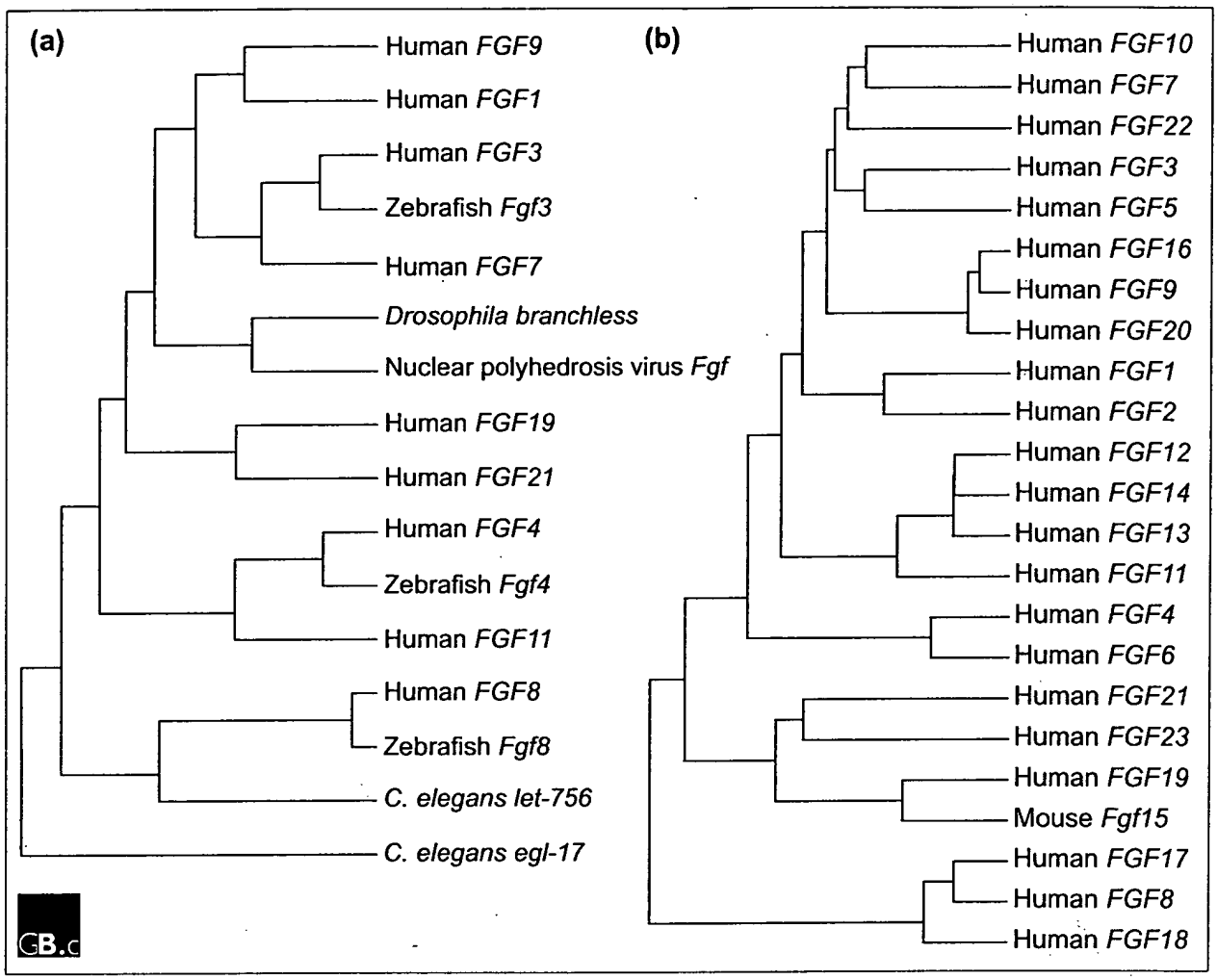

Figure 2
Evolutionary relationships within the FGF family. **(a)** Apparent evolutionary relationships between FGFs from vertebrates, invertebrates and a virus. Amino-acid sequences of nine representative FGFs were chosen from human and compared with FGFs from *Drosophila*, *C. elegans*, zebrafish and *Autographa californica* nuclear polyhedrosis virus. **(b)** Apparent evolutionary relationships of the 22 known human and murine FGFs. Sequences were aligned using Genetyx sequence analysis software and trees were constructed from the alignments using the neighbor-joining method.

Developmental expression patterns and function

The 22 members of the mammalian FGF family are differentially expressed in many, if not all, tissues, but the patterns and timing of expression vary. Subfamilies of FGFs tend to have similar patterns of expression, although each FGF also appears to have unique sites of expression. Some FGFs are expressed exclusively during embryonic development (for example, *Fgf3, 4, 8, 15, 17* and *19*), whereas others are expressed in embryonic and adult tissues (for example, *Fgf1, 2, 5-7, 9-14, 16, 18,* and *20-23*).

Function

The expression patterns of FGFs (see above) suggest that they have important roles in development. FGFs often signal directionally and reciprocally across epithelial-mesenchymal boundaries [36]. The integrity of these signaling pathways requires extremely tight regulation of FGF activity and receptor specificity. For example, in vertebrate limb development, mesenchymally expressed *Fgf10* in the lateral-plate mesoderm induces the formation of the overlying apical ectodermal ridge; the ridge subsequently expresses *Fgf8*, which signals back to the underlying mesoderm [37]. This directional signaling initiates feedback loops and, along with other signaling molecules, regulates the outgrowth and patterning of the limb. Importantly, the differential expression of the alternative splice forms of the receptors in the apical ectodermal ridge and underlying mesoderm is such as to limit or prevent autocrine signaling within a given compartment.

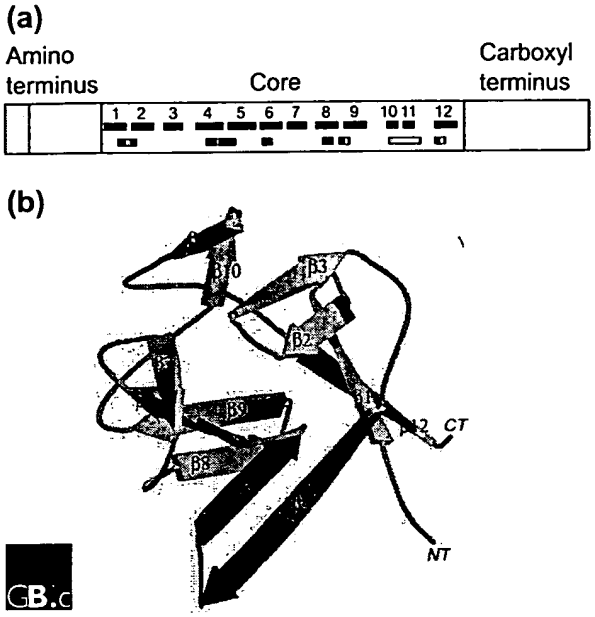

Figure 3
(a) Structural features of the FGF polypeptide. The amino terminus of some FGFs contains a signal sequence (shaded). All FGFs contain a core region that contains conserved amino-acid residues and conserved structural motifs. The locations of β strands within the core region are numbered and shown as black boxes. The heparin-binding region (pink) includes residues in the loop between β strands 1 and 2 and in β strands 10 and 11. Residues that contact the FGFR are shown in green (the region contacting Ig-domain 2 of the receptor), blue (contacting Ig-domain 3) and red (contacting the alternatively spliced region of Ig-domain 3). Amino-acid residues that contact the linker region are shown in gray [20]. **(b)** Three-dimensional structure of FGF2, a prototypical member of the FGF family. A ribbon diagram of FGF2 is shown; β strands are labeled 1-12 and regions of contact with the FGFR and heparin are color-coded as in (a) [22,24]. Image provided by M. Mohammadi.

Studies of the biochemical activities of FGFs have focused on the specificity of interactions between FGFs and FGFRs, on factors that affect the stability of FGFs and on the composition and mechanism of the active FGF-FGFR signaling complex.

Specificity of FGFs for FGF receptors

The FGFR tyrosine kinase receptors contain two or three immunoglobulin-like domains and a heparin-binding sequence [38-40]. Alternative mRNA splicing of the FGFR gene specifies the sequence of the carboxy-terminal half of immunoglobulin-domain III, resulting in either the IIIb or the IIIc isoform of the FGFR [41-43]. This alternative-splicing event is regulated in a tissue-specific manner and dramatically affects ligand-receptor binding specificity [44-48]. Exon IIIb is expressed in epithelial lineages and exon IIIc tends to be expressed in mesenchymal lineages [44,46-48]. *In vitro* patterns of binding specificity have been determined for each splice form of FGFR1-3 and for FGFR4, which is not alternatively spliced [49-51]. Ligands specific for these receptor splice forms are expressed in adjacent tissues, resulting in directional epithelial-mesenchymal signaling. For example, epithelially expressed FGFR2b (that is, FGFR2 IIIb isoform) can be activated by FGF7 and FGF10, ligands produced in mesenchymal tissue [49-51]. These ligands show no activity towards mesenchymally expressed FGFR2c. Conversely, FGF8 is expressed in epithelial tissue and activates FGFR2c but shows no activity towards FGFR2b ([49,52] and our unpublished observations). Notably, FGF8 expression is often restricted to epithelial tissue such as the apical ectodermal ridge of the developing limb bud [53,54].

Interaction with heparin or heparan sulfate proteoglycans

An important feature of FGF biology involves the interaction between FGF and heparin or heparan sulfate (HS) proteoglycan (HSPG) [19]. These interactions stabilize FGFs to thermal denaturation and proteolysis and may severely limit their diffusion and release into interstitial spaces [55,56]. FGFs must saturate nearby HS-binding sites before exerting an effect on tissue further away, or else must be mobilized by heparin/HS-degrading enzymes. The interaction between FGFs and HS results in the formation of dimers and higher-order oligomers [57-59]. Although the biologically active form of FGF is poorly defined, it has been established that heparin is required for FGF to effectively activate the FGFR in cells that are deficient in or unable to synthesize HSPG or in cells pretreated with heparin/HS-degrading enzymes or inhibitors of sulfation [60-62]. Genetic studies have also shown that mutations in enzymes involved in HS biosynthesis affect FGF signaling pathways during development [19,63]. Additional studies have shown that heparin and/or HS act to increase the affinity and half-life of the FGF-FGFR complex (reviewed in [40,64]).

A minimal complex containing one FGF molecule per FGFR can form in the absence of HS [24]. Structural studies suggest that HS may bridge FGF2 and the FGFR by binding to a groove formed by the heparan-binding sites of both the ligand and the receptor [24,65]. Binding studies with soluble chimeric FGFRs have identified a second potential FGF-binding site that, in some cases, can interact cooperatively with the primary FGF-binding site [66].

Important mutants

Many members of the *Fgf* family have been disrupted by homologous recombination in mice. The phenotypes range from very early embryonic lethality to subtle phenotypes in adult mice. The major phenotypes observed in *Fgf* knockout mice are shown in Table 2. Because FGFs within a subfamily have similar receptor-binding properties and overlapping patterns of expression, functional redundancy is likely to occur. This has been demonstrated for *Fgf17* and *Fgf8*, which cooperate to regulate neuroepithelial proliferation in the midbrain-hindbrain junction [17]. In the case of *Fgf* knockouts

resulting in early lethality, other functions later in development will need to be addressed by constructing conditional alleles that can be targeted at specific times and places in development. For example, *Fgf8* $^{-/-}$ mice die by embryonic day 9.5 [67]. A conditional allele for *Fgf8* targeted to the apical ectodermal ridge has been used to demonstrate an essential role for *Fgf8* in early limb development [68,69].

Several mutations in *Fgf* genes have been identified in *C. elegans*, *Drosophila*, zebrafish, mouse and human. The *C. elegans* gene *egl-17* is required for sex myoblast migration [12], and a null allele of *let-756* causes developmental arrest of the early larva [13]. The *Drosophila branchless* gene is required for tracheal branching and cell migration [11]. In zebrafish, *acerebellar (ace)* embryos lack the cerebellum and the midbrain-hindbrain boundary organizer. The *ace* gene encodes the zebrafish homolog of *Fgf8* [70]. Interestingly, zebrafish *aussicht* mutant embryos, which overexpress *Fgf8*, also have defects in development of the central nervous system [71].

In the mouse, the *angora* mutation, which affects hair growth, was found to be allelic with *Fgf5* [72]. A mouse mutant with a Crouzon-syndrome-like craniofacial dysmorphology phenotype was found to result from an insertional mutation in the *Fgf3/Fgf4* locus [73]. Recently, positional cloning of the autosomal dominant hypophosphataemic rickets gene identified missense mutations in human FGF23 [74]. A recent paper demonstrates that this disease is caused by a gain-of-function mutation [75]. The chromosomal location (Xq26) and tissue-specific expression pattern of *Fgf13* (also called *Fhf2*) suggests that it may be a candidate gene for Borjeson-Forssman-Lehmann syndrome, an X-linked mental retardation syndrome [76].

Frontiers

Issues most studied

FGFs have been intensely studied for nearly 30 years. Most of the early work focused on the mechanisms that regulate stability, secretion, export and interactions with heparin and on the mechanisms and consequences of signal transduction in various types of cells. More recent work has focused on the mechanisms regulating receptor specificity and receptor activation, the structure of the FGF-FGFR-HS complex, and the identification of new members of the FGF family. Functional studies have begun to address the role of FGFs in cell biology, development and physiology. Initial studies focused on the regulation of cell proliferation, migration and differentiation; more recent work has addressed the negative

Table 2

FGF knockout mice

Gene	Survival of null mutant*	Phenotype	References
Fgf1	Viable	None identified	[110]
Fgf2	Viable	Mild cardiovascular, skeletal, neuronal	[110-114]
Fgf3	Viable	Mild inner ear, skeletal (tail)	[115]
Fgf4	Lethal, E4-5	Inner cell mass proliferation	[116]
Fgf5	Viable	Long hair, angora mutation	[72]
Fgf6	Viable	Subtle, muscle regeneration	[117-119]
Fgf7	Viable	Hair follicle growth, ureteric bud growth	[120,121]
Fgf8	Lethal, E7	Gastrulation defect, CNS development, limb development	[67,70,122,123]
Fgf9	Lethal, P0	Lung mesenchyme, XY sex reversal	[124]; (J.S. Colvin *et al.*, personal communication)
Fgf10	Lethal, P0	Development of multiple organs, including limb, lung, thymus, pituitary	[125-127]
Fgf12 (Fhf1)	Viable	Neuromuscular phenotype	(J. Schoorlemmer and M. Goldfarb, personal communication)
Fgf14 (Fhf4)	Viable	Neurological phenotypes	(Q. Wang, personal communication)
Fgf15	Lethal, E9.5	Not clear	(J.R. McWhirter, personal communication)
Fgf17	Viable	Cerebellar development	[17]
Fgf18	Lethal, P0	Skeletal development	(N. Ohbayashi, Z. Liu, personal communication)

*E, embryonic day; P, postnatal day.

effect of FGFs and FGFRs on proliferation of some cell types, which was surprising as FGFs were thought to promote proliferation. *In vitro* studies have now been complemented by gene targeting in mice. The knockout approach has been fairly successful in identifying primary phenotypes but will be challenged by the need to address redundancy amongst the 22 FGFs and to study their developmental and physiological functions after the point of lethality of the null allele.

Unresolved questions

A major unresolved question concerns the mechanism(s) regulating FGF activity *in vivo* in the presence of cell-surface and extracellular-matrix HSPG. Current hypotheses predict that tissue-specific heparan fragments of defined sequence (and particularly of defined sulfation pattern) will differentially regulate FGFs by controlling their diffusion in the extracellular matrix and their ability to activate specific receptors [77]. These issues will be resolved by determining the sequence of tissue-specific HS and by demonstrating whether specific HS sequences can modulate the binding specificity of FGFs beyond that determined by the specific FGFR and its alternative splice form in the presence of heparin.

A second area of research will aim to elucidate the developmental roles of all the FGFs, first alone and then in various combinations. This will include determining whether a single FGF with a defined developmental function interacts with one or multiple FGFRs. A third major frontier will be to elucidate the physiological roles of FGFs that are expressed in adult tissues. This will again involve testing combinations of FGFs in cases in which knockouts are viable and designing conditional alleles in cases of embryonic lethality. Major areas being considered include neuronal and cardiovascular physiology, neuronal regeneration and homeostasis and tissue repair.

The last major frontier will be to elucidate the primary roles of FGFs in genetic diseases and cancer. Several FGFs were initially cloned from human and animal tumors. Future work will be required to determine whether FGF activation is itself an etiological agent in primary human tumors or whether it is a progression factor in the pathogenesis of cancer. As functional roles for FGFs are elucidated in embryonic development, it is expected that various human birth defects and genetic diseases will be attributed to mutations in *Fgf* genes. These studies will probably lead to the development of pharmacogenetic agents to treat these diseases. Because a large number of skeletal diseases are caused by mutations in *Fgfr* genes, it is anticipated that mutations in some *Fgf* genes will also be involved in skeletal pathology.

Acknowledgements

This work was supported by NIH grants CA60673 and HD35692 and by a grant from the American Heart Association (to D.M.O.) and by the Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan and a grant from the Human Frontier Science Program, France (to N.I.).

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